DNA :DNA Hybridization Studies of *Rhizobium japonicum* and Related *Rhizobiaceae*

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The symbiotic nitrogen-fixing bacteria associated with soybeans (*Glycine max*) have been classified routinely as *Rhizobium japonicum*. DNA :DNA hybridization studies were conducted to estimate the genetic relationships of strains labelled as *R. japonicum* to clarify their taxonomic status. Reassociation reactions were carried out using DNA from various *Rhizobium* and *Agrobacterium* species. Relationships of DNA sequences were also estimated by comparing the thermal stability of heterologous DNA duplexes with that of the homologous DNA duplex. The results indicated that strains labelled as *R. japonicum* can be separated into at least three DNA homology groups. Reference strains of other *Rhizobium* species, with the exception of *R. lupini*, were not closely related to these homology groups.

INTRODUCTION

The present classification of *Rhizobium*, the symbiotic nitrogen-fixing bacteria, is based on cross-inoculation between the bacteria and their various legume hosts and results in the designation of six species: *Rhizobium meliloti*, *R. trifolii*, *R. phaseoli*, *R. lupini*, *R. leguminosarum* and *R. japonicum*. This scheme, originally proposed by Fred *et al.* (1932), has been inadequate from the time of its acceptance due to inconsistencies. For example, *R. japonicum*, which was given a separate species status due to its nodulation of soybeans, was later found to infect some other legumes nodulated by other rhizobia. In addition, other groups were entirely excluded from classification by this system, such as the 'cowpea' group, which is promiscuous (Lange, 1961).

In the species *R. japonicum*, comparisons of the guanine + cytosine content in DNA have indicated a range of values from 61 to 64 mol% (Elkan & Usanis, 1971). However, within this relatively narrow range, strains could be arranged into at least two clearly separated clusters of values which, when converted to maximum percentage DNA homology, suggested the presence of two moderately divergent groups.

Relationships among strains may also be suggested by their specific antigenic properties. In this regard, the serological profile of *R. japonicum* indicated a high degree of antigenic heterogeneity (Date, 1962).

The results of some relatively recent genetic exchange experiments (Johnston & Beringer, 1977) and DNA :DNA hybridization studies (Gibbons & Gregory, 1972; Heberlein *et al.*, 1967; Jarvis *et al.*, 1980) support the need for reconsideration of the current *Rhizobium* classification. In this investigation, we have determined the DNA sequence relationships of strains labelled as *R. japonicum* and some 'cowpea' rhizobia.

Table 1. *Rhizobium* and *Agrobacterium* strains tested

Organism	Source*	Organism	Source*
<i>R. japonicum</i>		<i>R. trifolii</i>	
61A50	Nitragin Co., U.S.A.	ATCC 14480	ATCC
61A76	Nitragin Co., U.S.A.		
USDA 86	USDA	<i>R. meliloti</i>	
USDA 24	USDA	ATCC 9930	ATCC
USDA 29	USDA		
USDA 110	USDA	<i>R. leguminosarum</i>	
USDA 76	USDA	ATCC 10004	ATCC
USDA 140	USDA	ATCC 10314	ATCC
USDA 31	USDA		
USDA 46	USDA	<i>R. lupini</i>	
USDA 38	USDA	ATCC 10319	ATCC
USDA 123	USDA		
USDA 62	USDA	<i>R. phaseoli</i>	
USDA 94	USDA	127K14	Nitragin Co., U.S.A.
USDA 58	USDA	ATCC 14482	ATCC
USDA 115	USDA		
USDA 130	USDA	<i>Rhizobium</i> sp.	
USDA 122	USDA	32H1	Nitragin Co., U.S.A.
USDA 117	USDA	SMS-2	Inst. Agronomico, Brazil
5631	W. R. Grace Co., U.S.A.	3G4b4	USDA
5633	W. R. Grace Co., U.S.A.		
THA6	Texas A & M Univ., U.S.A.	<i>A. tumefaciens</i>	
8°	Iowa State Univ., U.S.A.	A80	Univ. N. Carolina, U.S.A.
505	Univ. Wisconsin, U.S.A.	B208	Univ. N. Carolina, U.S.A.
508	Univ. Wisconsin, U.S.A.		
ATCC 10324	ATCC	<i>A. radiobacter</i>	
D193	CRIPP, Czechoslovakia	6467	Univ. N. Carolina, U.S.A.
D209	CRIPP, Czechoslovakia		

* ATCC, American Type Culture Collection, Rockville, Md., U.S.A.; CRIPP, Central Research Institute for Plant Production, Prague, Czechoslovakia; W. R. Grace Co., Princeton, Ill., U.S.A.; Instituto Agronomico, Campinas, Brazil; Iowa State University, Ames, Iowa, U.S.A.; Nitragin Co., Milwaukee, Wis., U.S.A.; Texas A & M University, College Station, Tex., U.S.A.; University of North Carolina, Chapel Hill, N.C., U.S.A.; University of Wisconsin, Madison, Wis., U.S.A.; USDA, US Department of Agriculture, Beltsville, Md., U.S.A.

METHODS

Bacteria and growth conditions. The *Rhizobium* strains used in these studies were obtained from several sources (Table 1). The *R. japonicum*, 'cowpea' and *R. lupini* species were maintained by routine transfer on an agar medium containing HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid) and MES [2-(*N*-morpholino)ethanesulphonic acid], a variety of salts and gluconate, designated HMG (Cole & Elkan, 1973). The remaining *Rhizobium* species were grown on yeast extract mannitol (YEM) agar (Lillich & Elkan, 1968). *Agrobacterium* strains were maintained on nutrient agar and were cultured in nutrient broth medium. Stock slants were stored at 4 °C and transferred monthly to fresh slants. The *R. japonicum* strains were also cultured on solid medium plates for confirmation of colony morphologies. Production of nodules on legumes was verified for each of the *Rhizobium* species used. The authenticity of the *R. japonicum* strains was further verified by phage typing.

For inocula, the *R. japonicum* strains were grown in 10 ml HMG broth in 50 ml DeLong flasks and were incubated on a gyrotary shaker at 28 to 30 °C for 4 d. Two transfers in a similar medium were made before the cultures were used as inocula. Negative growth response on LB medium (Miller, 1972) was used as a control for culture purity: this medium contains 1% NaCl to inhibit growth of rhizobia.

Media and stock solutions. The HMG broth medium employed for labelling DNA of cells contained carrier-free $\text{H}_3^{32}\text{PO}_4$ (5 mCi l⁻¹). The HMG broth is a synthetic buffering system unaffected by deletion or addition of small quantities of phosphates. The medium was adjusted to pH 6.8 to 7.0 prior to sterilization. The radioisotope was added to inoculated media at early-exponential phase (48 h growth) during incubation at 28 to 30 °C. Cultures were then shaken in the presence of the radioisotope for an additional 48 h. Unlabelled cells were grown in HMG broth for a total of 4 d under similar conditions to labelled cells but without the addition of radioisotope.

Cell lysis and isolation of DNA. Cell lysis was initiated by the addition of sodium dodecyl sulphate (final concentration 2%, w/v) to 25 ml of buffered saline-EDTA (0.15 M-NaCl, 0.1 M-EDTA, pH 8.0) cell suspension. The suspension was placed in a 60 °C water bath for 10 min, followed by cooling at room temperature. The DNA released was isolated and purified by a modification of the procedures of Brenner *et al.* (1969) as reported by Kloos & Wolfshohl (1979).

DNA reassociation reactions. In most experiments, 0.1 µg of sheared, heat-denatured, ³²P-labelled DNA was allowed to react with 120 µg of sheared, heat-denatured, unlabelled DNA in 1 ml of 0.28 M-phosphate buffer. (PB, a buffer solution containing equimolar quantities of Na₂HPO₄ and NaH₂PO₄) at 65 or 80 °C (non-restrictive and restrictive conditions, respectively). At the termination of reactions, unreacted, single-stranded DNA was separated from reassociated, double-stranded DNA on hydroxyapatite by the batch procedure described by Brenner and co-workers (1969, 1972, 1976). The concentration of unlabelled DNA and reaction incubation time were selected to provide nearly maximal reassociation of labelled DNA with unlabelled DNA (56 ± 5% in homologous reactions). The concentration of labelled DNA was small enough to prevent significant reassociation of labelled DNA fragments with one another (<4%). The percentage relative binding was determined by normalizing the amount of DNA bound on hydroxyapatite in heterologous reactions to that bound in homologous reactions. For determining the thermal stability of DNA duplexes, reassociated DNA prepared at 65 °C and adsorbed on hydroxyapatite was eluted in a series of 0.14 M-PB washes at increasing 2.5 °C increments from 65 to 100 °C to produce a thermal elution profile. This profile was then used to determine the midpoint of thermal elution ($T_{m(e)}$) of the hybridized DNA.

RESULTS

In order to clarify the genomic relationships of strains labelled as *R. japonicum* species, DNA:DNA hybridization studies were carried out to determine the relative binding of heterologous DNAs and thermal stability of DNA duplexes. Comparisons were also made between *R. japonicum* strains, other *Rhizobium* species and *Agrobacterium* species. Although the various *R. japonicum* strains were isolated from many geographical areas, the results indicated that the source of the various organisms was completely independent of the degree of genetic relatedness revealed throughout the reassociation experiments. As can be seen from the reassociation data (Table 2), strains labelled as *R. japonicum* fall into at least three distinct DNA homology groups. One of these groups, designated *R. japonicum* subspecies 1 or homology group I, contains both the *R. japonicum* reference strain ATCC 10324 and the *R. lupini* reference strain ATCC 10319, together with nine other strains labelled as *R. japonicum*. Another group, designated *R. japonicum* subspecies 2 or homology group Ia, containing *R. japonicum* USDA 110 and five other strains, is more closely related to homology group I (relative binding 51 ± 7% at 65 °C, 35 ± 9% at 80 °C) than to the third group (relative binding 29 ± 7% at 65 °C, 11 ± 5% at 80 °C) designated *Rhizobium* sp. or homology group II. The widely divergent homology group II is composed of *R. japonicum* 61A76 and eight other strains. Several other strains labelled as *R. japonicum* and the 'cowpea' rhizobia have diverged significantly and may represent yet additional DNA homology groups.

Thermal elution profile data (not shown) indicated that the unpaired bases in duplexes formed between DNA of the reference strain *R. japonicum* ATCC 10324 and DNAs of *R. japonicum* D193 and *R. lupini* ATCC 10319 were no more than 1.0% of the total genome assayed. In contrast, the thermal elution profiles of heterologous DNA duplexes formed between *R. japonicum*, ATCC 10324 and USDA 31, 'cowpea' 32H1 and 61A76 displayed a $T_{m(e)}$ averaging over 10 °C below that of the homologous *R. japonicum* ATCC 10324 DNA duplex, suggesting that more than 10% of the bases were mismatched; this is consistent with a separate species status. The moderate decrease in thermal stability ($\Delta T_{m(e)} = 6$ °C) observed for heterologous DNA duplexes formed by the reassociation of DNA of *R. japonicum* strains ATCC 10324, USDA 110 and 8° may indicate an intermediate or subspecies relationship between *R. japonicum* ATCC 10324 of DNA homology group I and the above *R. japonicum* strains of DNA homology group Ia. The thermal stability results thus complemented the relative binding data used to delineate DNA homology groups.

DISCUSSION

The separation of strains labelled as *R. japonicum* into at least three DNA homology groups shows the diversity of these bacteria and casts serious doubt on the validity of a single species designation for this group.

Hybridization data for *Rhizobium* strains 3G4b4 and SMS-2, both 'cowpea' rhizobia, and *R. japonicum* 505 fall within the possible limits of a borderline species or subspecies relationship to *R. japonicum* (DNA homology groups I and Ia). The above 'cowpea' strains are not as closely related to the 'cowpea' strain 32H1. It would appear that 'cowpea' rhizobia, like strains labelled as *R. japonicum*, may represent at least two different species. Due to the limited number of agrobacteria included in this study, it is difficult to evaluate the overall degree of similarity between *Agrobacterium* and *Rhizobium*. The three *Agrobacterium* strains examined demonstrated very little relationship to the rhizobia selected as sources of labelled DNA. Graham's (1964) data indicated a slight relatedness between the fast-growing rhizobia and *Agrobacterium*.

It is interesting to note that the *R. lupini* reference strain ATCC 10319 and *R. japonicum* reference strain ATCC 10324 are highly related as determined by reciprocal DNA reassociation reactions and the thermal stability of DNA duplexes. The *R. lupini* strain used is also closely related to several other *R. japonicum* strains (USDA 24, D209, USDA 123). Since only the single reference *R. lupini* strain was included in this study, we do not wish to

Table 3. Comparison of DNA homology and serological groups of strains labelled as *Rhizobium japonicum*

	DNA homology group	Serological group*
	Group I	
<i>R. japonicum</i> subspecies 1	ATCC 10324†	24
	USDA 24	24
	D193	24
	D209	24
	THA6	76
	USDA 58	58
	USDA 123	123
	USDA 38	38
	5633	123
	USDA 115	38-115
	Group Ia	
<i>R. japonicum</i> subspecies 2	USDA 110	110
	8°	110
	USDA 140	62
	5631	110
	USDA 62	62
	61A50	110
	Group II	
<i>Rhizobium</i> sp.	61A76	31
	USDA 86	86
	USDA 76	76
	USDA 29	31
	USDA 46	46
	USDA 31	31
	USDA 117	76
	USDA 130	130
	USDA 94	94

* According to Date (1962).

† *R. japonicum* reference strain.

conclude now that *R. lupini* and *R. japonicum* should be combined in one species. An earlier suggestion that these species should be combined was made by Graham (1964) based on numerical taxonomic data. This view was consistent with the DNA base composition data of De Ley & Rassel (1965). Other DNA:DNA hybridization studies (Gibbons & Gregory, 1972) showed that several strains of *R. lupini* and *R. japonicum* were very closely related. We are expanding our studies to determine whether these two species should be combined or if perhaps the *R. lupini* reference strain is atypical of the species.

The fact that strains labelled as *R. japonicum* displayed considerable diversity in their nucleotide sequence relatedness becomes an important issue in *Rhizobium* research since results determined for a particular test strain may show considerable departure from the reference *R. japonicum* strain ATCC 10324. It is significant that many of the strains of *R. japonicum* fell into three different DNA homology groups and that two of these groups are so widely divergent that they probably represent different species. Originally, all of the *R. japonicum* strains used in this study were authenticated in nodulation tests, so on the basis of the 'cross-inoculation' concept and prevailing criteria, they were named appropriately. Obviously, the taxonomic status of *R. japonicum* needs re-evaluation. With only one possible exception, *R. japonicum* THA6, members of the same serological group are included in the same DNA homology group (Table 3). Should serological type remain a valid character predicting DNA relatedness in future work, this would afford ecologists with a simple test for identifying *Rhizobium* species.

Our results strongly indicate the inadequacy of the current classification of rhizobia based on cross-inoculation between the bacteria and their host legume. Not only does it appear that there is more than one species designated as *R. japonicum* but there is also an indication that *R. lupini* may not be a species different from *R. japonicum*. On the strength of these data, we have begun an extensive re-examination of the taxonomy of *Rhizobium* using DNA:DNA hybridization techniques.

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REFERENCES

- BRENNER, D. J., FANNING, G. R., RAKE, A. V. & JOHNSON, K. E. (1969). Batch procedure for thermal elution of DNA from hydroxyapatite. *Analytical Biochemistry* **28**, 447-459.
- BRENNER, D. J., FANNING, G. R., SKERMAN, F. J. & FALKOW, S. (1972). Polynucleotide sequence divergence among strains of *Escherichia coli* and closely related organisms. *Journal of Bacteriology* **109**, 953-965.
- BRENNER, D. J., STEIGERWALT, A. G., FALCAE, D. P., WEAVER, R. E. & FANNING, G. R. (1976). Characterization of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* by deoxyribonucleic acid hybridization and by biochemical reactions. *International Journal of Systematic Bacteriology* **26**, 180-194.
- COLE, M. A. & ELKAN, G. H. (1973). Transmissible resistance to penicillin G, neomycin, and chloramphenicol in *Rhizobium japonicum*. *Antimicrobial Agents and Chemotherapy* **4**, 248-253.
- DATE, R. A. (1962). *Root nodule bacteria and legume relationships: studies on serological classification, competition effects, and survival and location in preinoculated seed*. Doctoral thesis. Department of Bacteriology, University of Maryland, U.S.A.
- DE LEY, J. & RASSEL, A. (1965). DNA base composition, flagellation and taxonomy of the genus *Rhizobium*. *Journal of General Microbiology* **41**, 85-91.
- ELKAN, G. H. & USANIS, R. A. (1971). Theoretical deoxyribonucleic acid homology between strains of *Rhizobium japonicum*. *International Journal of Systematic Bacteriology* **21**, 295-298.
- FRED, E. B., BALDWIN, I. L. & MCCOY, E. (1932). *Root Nodule Bacteria and Leguminous Plants*. Madison: University of Wisconsin Press.
- GIBBONS, A. M. & GREGORY, K. F. (1972). Relatedness among *Rhizobium* and *Agrobacterium* species determined by three methods of nucleic acid hybridization. *Journal of Bacteriology* **111**, 129-141.
- GRAHAM, P. H. (1964). The application of computer techniques to the taxonomy of the root nodule bacteria of legumes. *Journal of General Microbiology* **35**, 511-517.
- HEBERLEIN, G. T., DE LEY, J. & TITGAT, R. (1967). Deoxyribonucleic acid homology and taxonomy of *Agrobacterium*, *Rhizobium*, and *Chromobacterium*. *Journal of Bacteriology* **94**, 116-124.

- JARVIS, B. D. W., DICK, A. G. & GREENWOOD, R. M. (1980). Deoxyribonucleic acid homology among strains of *Rhizobium trifolii* and related species. *International Journal of Systematic Bacteriology* **30**, 42–52.
- JOHNSTON, A. W. B. & BERINGER, J. E. (1977). Chromosomal recombination between *Rhizobium* species. *Nature, London* **267**, 611–613.
- KLOOS, W. E. & WOLFSHOHL, J. F. (1979). Evidence for deoxyribonucleotide sequence divergence between staphylococci living on human and other primate skin. *Current Microbiology* **3**, 167–172.
- LANGE, R. T. (1961). Nodule bacteria associated with the indigenous leguminosae of South-Western Australia. *Journal of General Microbiology* **26**, 351–359.
- LILLICH, T. T. & ELKAN, G. H. (1968). Evidence countering the role of polygalacturonase in invasion of root hairs of leguminous plants by *Rhizobium* spp. *Canadian Journal of Microbiology* **14**, 617–625.
- MILLER, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory.